

# The use of quantitative Real-Time PCR to estimate Salmonella shed in fecal samples from naturally infected finishing pigs

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## Abstract

The objective of this study was to describe the shedding pattern of Salmonella in finishing pigs, as well to quantify the Salmonella load. A longitudinal study was conducted in 12 cohorts of pigs in a multi-site farrow-to-finish production system. At the beginning of each cohort, 50 pigs ( $10 \pm 2$  weeks old) were randomly selected and individually identified. Individual pig fecal samples were collected and cultured every 2 weeks for 16 weeks (8 collections). Further, quantitative real-time PCR (q-PCR) targeting the *invA* gene was performed in a subset of the culture negative samples (555) and in all available culture positive samples. At the time of submission, Salmonella was cultured from 397 (8.74%) of 4540 individual fecal samples. Overall incidence of Salmonella was 24.8% (149/600 pigs). The proportion of positive samples decreased over the finishing period from 16.75% (10 weeks old) to 4.30% (24-26 weeks old). At the present, all Salmonella culture negative samples subjected to q-PCR were PCR negative. Of culture positive samples, 16% were detected by q-PCR, and only 3 % of the samples were within the quantifiable range of detection ( $>10^3$  colony forming units per gram of feces). Of those samples within the quantifiable range, the bacterial concentration ranged from  $1.05 \times 10^3$  to  $1.73 \times 10^6$  *invA* gene copies/g feces. The results suggest that point estimates of Salmonella prevalence may not accurately describe the Salmonella status of finishing pigs. The majority of pigs shed Salmonella at low concentrations. These preliminary data can contribute to quantitative risk assessments of the association between concentrations of Salmonella shed by pigs during the finishing phase and contributions to carcass contamination at slaughter.

## Introduction

Salmonella is one of the major causes of bacterial foodborne disease in United States (Henao et al., 2011). Salmonella is one the most important bacteriological zoonotic hazards transmissible from pork to consumers (Boyen et al., 2008). Reduction of the Salmonella contamination of pork and pork products requires interventions at pre-harvest, harvest and post-harvest (Lo Fo Wong et al., 2002). Longitudinal studies have shown high variability in Salmonella shedding over time at both the farm and individual level (Funk et al., 2001b; Rajic et al., 2005). Therefore, there is a need to better describe Salmonella shedding in order to understand Salmonella dynamics and implement control measures to reduce Salmonella pre-harvest. Limited research has been conducted to quantify Salmonella load in naturally infected finishing pigs. Enumeration of bacterial load can be used to identify contamination pressure and to implement effective control measures to reduce contamination (Fravalo et al., 2003; Boughton et al., 2007). In addition, quantitative data is needed for quantitative microbial risk assessments (QMRA) and modeling transmission patterns of Salmonella (Bollaerts et al., 2009; Lanzas et al., 2011). The objective of this study was to describe the shedding pattern of Salmonella in finishing pigs, as well to quantify the Salmonella load.

## Materials and Methods

A longitudinal study was conducted in three sites of a multi-site farrow-to-finish production system. At each 4-barn site (A, B, C) one barn was selected for study inclusion. For each barn selected six (sites A & B) and two (site C) consecutive cohorts of pigs were included in the study. At the beginning of each cohort, 50 pigs ( $10 \pm 2$  week old) were randomly selected and individually identified. Individual pig fecal samples (10g) were collected and cultured every 2 weeks for 16 weeks (8 total sampling periods per cohort). Fecal samples were culture using standard enrichment adapted from Davies et al. (2000) for 10 gram fecal weights (TTB, RV, XLT-4). An additional fecal sample from each pig (200mg) was kept frozen ( $-80^\circ\text{C}$ ) for later analysis using quantitative real-time PCR (q-PCR). A random selection of culture negative samples ( $n=555$ ) and all the available culture positive samples ( $n=381$ ) were submitted for real-time q-PCR targeting the *invA* gene. The

DNA was extracted from 200 mg of feces using the Qiagen Qiamp Stool Mini Kit (Qiagen, Valencia, CA) according to manufacturer instructions. The q-PCR was performed in triplicate for each sample of DNA using PerfeCTa qPCR SuperMix, low Rox (Quanta Biosciences, MD). The PCR primers and probe used for amplification and detection were described by Hoorfar et al. (2000). The limit of detection of the q-PCR was determined using sterilized fecal samples spiked with serial 10 fold dilutions of Salmonella. The concentration of Salmonella at the endpoint of detection in triplicate was confirmed by culture and colony count to be 103 CFU per gram of feces. A pig was Salmonella-positive if at least one fecal sample tested culture positive. Descriptive statistics of bacteriologic culture were estimated at the sample and pig level. Salmonella apparent prevalence (proportion of positive samples) and respective 95% confidence intervals were determined for each site, season, pig age and overall. Pearson Chi-squared analysis with Bonferroni adjustment was used to compare apparent prevalence among sites and season. Chi-squared test for trend in proportions (Cochran-Armitage Test) was applied to test change over time. A significance level of 0.05 was considered for comparisons (SAS 9.2). Descriptive statistics of q-PCR results were presented as copy numbers of *invA* gene/gram of feces and a scoring system which combined results of culture and q-PCR.

## Results

At the time of submission 12 cohorts are completed. All cohorts were Salmonella-positive in at least one fecal sampling. The range of positive samples per cohort was 1 to 156 samples. Salmonella was cultured from 8.74% (397 /4540; 95% C.I. 7.96-9.60%) individual fecal samples. Overall incidence of Salmonella was 24.8% (149/600 pigs; 95% C.I. 21.54-28.45%). Of positive pigs (149), 60 were positive once, 22 were positive twice, 26 pigs were positive three times, 16 four times and 25 pigs were positive five or more times during the sampling period. Among the positive pigs (89) that were detected positive in more than 2 sampling occasions, 42.69% (38/89) had consecutive positive culture samplings, 25.84% (23/89) had one culture negative fecal sample within the period of shedding and 31.46% (28/89) were culture negative in more than 2 occasions between two culture positive sample collection periods.

There was a significant difference between sites in the overall relative proportion of positive samples (p-value <0.05). Site B (12.71%; 95% C.I. 11.26 -14.33%) had higher proportion than site A (7.26%; 95% C.I. 6.18-8.47%) and site C (3.21%; 95% C.I. 2.14-4.62%). There was a significant difference in the overall relative proportion of positive samples between seasons. Fall (14%; 95% C.I. 12.04-16.23%) and winter (13.03%; 95% C.I. 11.33-14.92%) had a significantly higher proportion of positive samples when compared with spring (1.88%; 95% C.I. 1.09-3.16%) or summer (4.34%; 95% C.I. 3.39-5.55%). The proportion of positive samples decreased significantly over time (p-value <0.0001). The apparent prevalence decreased from 16.75% (95% C.I. 13.96-19.96%) at the beginning of the finishing period (10 weeks old) to 4.30% (95% C.I. 2.76-6.59%) at end (24-26 weeks old) (p-value <0.0001).

At the present, all of the Salmonella culture negative samples subject to q-PCR were PCR negative. Of culture positive samples, 16% (61/381) were detected by q-PCR, but only 3% (13/381) of the samples were detected in triplicate and were within the quantifiable range (>103). Individual samples were classified in 4 scores based on the detection limit of q-PCR, culture and q-PCR results. Those scores were combined in order to estimate a gradient of concentration. The four scores were: a) score 0: culture-negative and q-PCR-negative (555); b) score 1: culture-positive and q-PCR negative (321); c) score 2: culture and q-PCR positive, not within quantifiable range (48); d) score 3: culture positive and q-PCR positive within quantifiable range (13). The concentration gradient was assumed to increase from score 1 to score 3. In score group 3, the concentration ranged from 1.06x10<sup>3</sup> to 1.73x10<sup>6</sup> copies of the *invA* gene/gram feces. Fifty three percent (7/13) of the score 3 group samples belonged to the same pig. Fifty-seven percent (4/7) of the pigs in score 3 group were from the same cohort and pen.

## Discussion

The overall prevalence reported in this study is higher than others that have targeted the finishing phase in the US (Wang et al., 2010; Rostagno et al., 2011). Several explanations can be in base such as farm factors, study design, sampling strategy and diagnostic test (Rajic et al., 2005). The intensive repeated sampling conducted in this longitudinal study might have increased the probability of detecting positive pigs.

There was high variability in the prevalence among cohorts and sites, identical findings were described by others researchers. Therefore, point estimates might misclassify Salmonella status of farms or pigs (Funk et al., 2001b; Kranker

et al., 2003; Rajic et al., 2005). There was a trend of decreasing prevalence as the pigs got older. The prevalence was higher during the fall and winter, and seasonality on shedding has been reported by other authors (Funk et al., 2001a).

There was intermittent detection of shedding in almost fifty percent of the pigs with multiple culture-positive collections. Intermittent shedding has been reported in experimental and epidemiologic studies. Salmonella carriers can shed intermittently and for long periods (Funk et al., 2001b; Kranker et al., 2003; Scherer et al., 2008). Intermittent shedding could be an intermittent detection of an on-going infection or a new infection after clearance of a previous infection. The diagnostic sensitivity of culture ranges from 10% to 80%, depending on the protocol (Rajic et al., 2005). The imperfect sensitivity of the culture methods may affect the detection of the active shedders, thus some of those culture-negative samplings could be a false negative. Although a relative short sampling interval (15 days) was conducted in this study, new infections could occur between sampling occasions.

The majority of the pigs shed low concentrations, below the quantitative limit of qPCR. In a quantitative study using mini-MSRV, 97% of swine fecal samples had less than 200 organisms per gram (Fravalo et al., 2003). Quantitative studies at the lairage have reported variable and relative low bacterial load, median pen concentration ranging from 1.8-11.5 organisms/100cm<sup>2</sup> (Boughton et al., 2007) and 457-1071 organisms/ml (O'Connor et al., 2006) respectively. However, the findings between lairage and individual pigs are not directly comparable because of different sampling methodologies and the likely cumulative contamination of lairage. Naturally infected pigs tend to shed low numbers of Salmonella in feces. Only a small percentage of samples had higher concentrations and seemed to be clustered within cohort and pig.

## Conclusion

This interim report of an ongoing study suggests that point estimates might underestimate prevalence, and at least in this study, the majority of culture positive pigs shed Salmonella in low concentrations.

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